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The effects of radiofrequency electromagnetic radiation on sperm function.

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Short title: Impact of RF-EMR on spermatozoa

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Abstract

Mobile phone usage has become an integral part of our lives. However, the effects of the radiofrequency electromagnetic radiation (RF-EMR) emitted by these devices on biological systems and specifically the reproductive systems are currently under active debate. A fundamental hindrance to the current debate is that there is no clear mechanism of how such non-ionising radiation influences biological systems. Therefore, we explored the documented impacts of RF-EMR on the male reproductive system and considered any common observations that could provide insights on a potential mechanism. Among a total of 27 studies investigating the effects of RF-EMR on the male reproductive system, negative consequences of exposure were reported in 21. Within these 21 studies, 11 of the 15 that investigated sperm motility reported significant declines, 7 of 7 that measured the production of reactive oxygen species documented elevated levels and 4 of 5 studies that probed for DNA damage highlighted increased damage, due to RF-EMR exposure. Associated with this, RF-EMR treatment reduced antioxidant levels in 6 of 6 studies that studied this phenomenon, while consequences of RF-EMR were successfully ameliorated with the supplementation of antioxidants in all 3 studies that carried out these experiments. In light of this, we envisage a two-step mechanism whereby RF-EMR is able to induce mitochondrial dysfunction leading to elevated ROS production. A continued focus on research which aims to shed light on the biological effects of RF-EMR will allow us to test and assess this proposed mechanism in a variety of cell types.

1. Introduction

Over the past 20 years, the use of mobile phones has increased exponentially (Gorpinchenko *et al.*, 2014), with a current estimate of more than one billion users worldwide (French *et al*., 2001; Meral *et al*., 2007). In the United States there is approximately one device in use per person, and well above more than one person in European countries such as Germany, Denmark and Italy (U.S. Census Bureau, 2012). Furthermore, the number of devices in service is rising at an estimated rate of 3% annually (ACMA, 2013). Accordingly, the exposure of humans to radiofrequency electromagnetic radiation (RF-EMR) emitted from these devices has also increased substantially, with an average talk time of 30 min per day spent talking on mobile phones (CTIA, 2011). The effect of this radiation on human health remains to be fully elucidated with current literature detailing an array of apparently contradictory results. Indeed, while some studies have identified pronounced deleterious effects of RF-EMR on a variety of cell types (d'Ambrosio *et al.,* 2002; Balode, 1996; Bilgici *et al*., 2013; Dasdag *et al*., 2015; Furtado-Filho *et al*., 2014; Hou *et al*., 2014; Kahya *et al*., 2014), others have reported only very subtle or no significant impacts (Dasdag *et al*, 2009; Demirel *et al*., 2012; Khalil *et al*., 2014; Marchionni *et al*, 2006; Masuda *et al*., 2006). A confounding factor in these studies involves the use of differing RF intensity, frequency, exposure length and method of administration that discount the possibility of direct and robust study-to-study comparisons. Such variation attempts to simulate elevated levels of exposure in certain studies and real-life mobile phone exposure in others, which is extremely hard to model given the variability that exists in each of these parameters of intensity and frequency (Lerchl, 2013). For instance, the intensity of RF-EMR emitted from mobile phones varies from ~0.1 - 4 W/kg (La

Vignera *et al*., 2012; Fejes *et al.,* 2005; Guney *et al*., 2007), while mechanistic studies have involved intensities as high as 27.5 W/kg (De Iuliis *et al*., 2009a). Regardless of these differences, the balance of evidence supports the principle that RF-EMR has the ability to induce cellular damage (Adams *et al*., 2014). In light of this conclusion and to work toward identifying real clinical risks, it is imperative that we develop an understanding of the mechanism(s) by which this form of radiation affects different biological systems.

1.1 Physical parameters of RF-EMR

Radiofrequency-electromagnetic radiation is a form of microwave radiation, important properties of which include the frequency at which it is generated, measured in megahertz (MHz) or gigahertz (GHz), and the intensity of the waves, or the specific absorption rates (SAR); the energy carried as a quantity with respect to mass in watts per kilogram. The transfer of energy from the electromagnetic field to particles in an absorber is measured by the SAR, which indicates the quantity of energy related to mass, defined at a particular point in the absorber (Durney, 1986). The frequency of RF-EMR emitted by mobile phone devices is in the range of 900 to 1800 MHz and the intensity of this radiation is generally restricted to a local limit of <2 W/kg and whole-body limit of 0.08 W/kg (Chen, 2007; Durney, 1986) to enforce safe exposure levels in humans. Meanwhile, the ability of RF-EMR itself to penetrate into the skin and body is dependent on the permittivity and conductivity of the irradiated tissue, as well as the wavelength of the radiation, which is inversely related to the wave frequency (Figure 1). Therefore, at lower frequencies the penetration of the RF-EMR is further and devices operating in the 900 MHz range will irradiate the body more; approximately 25% of the body in humans compared to 20% penetration at 1800 MHz (Durney, 1986). However, it is possible that the penetration of RF-EMR

into the testis may be more pronounced than other tissues, due to the fact that this organ is less protected by tissue in comparison to others. Mobile phone communications uses a variety of different frequency ranges, with the most common utilising the 880-915MHz range for the global system for mobile communications (GSM) 900 uplink (from mobile phone to base station), 925-960 MHz for the GSM900 downlink (from base station to mobile phone), 1710-1785MHz for the DCS1800 uplink, 1805MHz-1880MHz for the GSM1800 downlink, 1920-1980MHz for the universal mobile telecommunications system (UTMS) data uplink and 2110-2170MHz for the UTMS data downlink (Bolte & Eikelboom, 2012). Of particular interest is this radiofrequency range, in which a majority of studies have utilized exposure frequencies of 900-1800 MHz. This in turn forms the basis of studies selected for this review.

1.2 Review focus

For the purpose of this review, we shall focus on an analysis of RF-EMR impacts on the male reproductive system, a site that may be uniquely vulnerable to chronic EMR exposure from devices stored in the vicinity of the testes that are held in ‘standby mode’ and, more importantly, at the initiation of a call or when hands-free mode is in use. Our specific interest is to draw a consensus regarding the impact of RF-EMR on the male germ line, with an emphasis on frequencies that equate to analog/digital signals (900/1800 MHz [Irmak *et al.*, 2002]) and with specific absorption rates (SAR) of up to 4 W/kg. We imposed strict search criteria which gives this review focus on probing a potential mechanism of action, independent of clinical significance. To source the appropriate studies, we utilized search terms of “rf-emr spermatozoa”; “radiofrequency electromagnetic radiation spermatozoa” and “cell phone radiation +

spermatozoa" in the PubMed database. Of those studies identified, we elected to review those reporting exposure at the RF range of between ~900-1800 MHz and that focused on the male reproductive tract / spermatozoa. Such criteria were imposed to reflect the intensity of radiation emitted from devices. This narrowed the list of articles to those summarised in Table 1. Largely independent of clinical significance, the unique cell biology of spermatozoa provides an ideal model in which the specific physical and chemical responses to EMR can be observed. These cells provide a sensitive model as (Aitken, 2013; Aitken *et al*., 2014): (i) they are sensitive to damage by environmental factors including free radicals, (ii) they can be maintained for 48-72 hours *in vitro* in simple, defined culture media, (iii) their motility provides a readily assessable means of monitoring adverse biological effects and (iv) they are clinically important, since DNA damage in spermatozoa has the potential to influence the health and wellbeing of the offspring. As a consequence of the information summarized in this review, we propose a mechanism for the negative effects of RF-EMR on the male germ line. Given the unique susceptibility of spermatozoa to subtle oxidative insults, which may arise from RF-EMR exposure, the translation toward clinical significance, especially involving other cell types, should not be made. However, given that spermatozoa may be acutely sensitivity to such stressors as RF-EMR, we propose that a clear hypothesis for a mechanism of action can be developed utilizing this model, which can then be applied for testing in other cell types.

2. The impact of RF-EMR on semen quality

Mobile phone use is becoming increasingly popular worldwide, with specific population groups, including businessmen and adolescents, estimated to spend as

much as half of their day in close proximity to mobile phones held in either active or standby modes (Redmayne *et al*., 2011; Roberts *et al*., 2014). Owing to the common practice of storing mobile phones in close proximity to the testes, these individuals may be unintentionally exposing their reproductive system to relatively high levels of RF-EMR. It is therefore of considerable concern that the use of mobile phones (Agarwal *et al*., 2009; Fejes *et al*., 2005; Gorpinchenko *et al*., 2014; Yan *et al*., 2007; Zalata *et al*., 2015), or exposure to RF-EMR emitted by these devices (Al-Damegh, 2012; De Iuliis *et al*., 2009a; Ghanbari *et al*., 2013), has been linked to negative impacts on semen quality. Notwithstanding considerable controversy regarding the timing and nature of such exposures (Dasdag *et al*., 2003; Imai *et al*., 2011; Tumkaya *et al*., 2013), the principle that RF-EMR can elicit a detrimental impact on sperm function is supported by a growing number of studies (Agarwal *et al*., 2009; De Iuliis *et al,* 2009a; Fejes *et* al., 2005; Gorpinchenko *et al*., 2014; Liu *et al*., 2013a, b; Mailankot *et al*., 2009). In general, these data lend support to the notion that RF-EMR can significantly impair key aspects of sperm function including the motility and vitality of these cells and the integrity of their DNA (Table 1), suggesting a direct effect on mature spermatozoa. However, there is less compelling evidence to suggest an additional role at the level of spermatogenesis in reducing sperm counts *in vivo* (Imai *et al.,* 2011; Tas *et al*., 2014). Indeed, a chronic, multi-generational study demonstrated RF-EMR to have no effects on sperm production, testicular or epididymal weight (Sommer *et al*., 2009).

Direct effects of RF-EMR on spermatozoa

In one of the earliest studies on the impact of RF-EMR on sperm quality, Wdowiak (*et al*., 2007) demonstrated that males who use mobile phones exhibit increased rates of abnormal sperm morphology and decreased motility compared to

counterparts that did not use these devices. Furthermore, these effects were exacerbated with longer exposure to this form of radiation (Wdowiak *et al*., 2007). Since this report, additional studies have replicated the adverse impact of RF-EMR treatment on human sperm motility utilising a model waveguide device capable of emitting finely tuned electromagnetic radiation to mimic that emitted by mobile phones (De Iuliis *et al*., 2009a; Gajda *et al*., 2002). The waveguide approach improves control of exposure as well as replicating the use of a mobile phone held in talk mode (Agarwal *et al*., 2009).

Males experiencing subfertility, for example asthenozoospermia and oligozoospermia, appear to be particularly vulnerable to RF-EMR as highlighted by a marked decline in sperm motility following exposure of semen samples to a mobile device for just 10 minutes (Zalata *et al*., 2015). Similar pronounced effects have also been documented following *in vivo* exposure of whole animals to a mobile phone operating in talk mode (Mailankot *et al*., 2009; Yan *et al*., 2007). In terms of the nature of the impaired motility, RF-EMR appears to impact primarily on the capacity of spermatozoa to sustain forward progressive motility. Indeed, a study by Erogul and colleagues (2006), confirmed that the exposure of human spermatozoa to RF-EMR compromised their ability to sustain both rapid and slow progressive motility after an alarmingly brief exposure time of only five minutes. While other studies have required longer exposure times (hours or days) to generate significant reductions in sperm motility, impaired progressive motility (involving a decrease in the percentage of cells displaying rapid progressive motility and a corresponding increase in cells expressing slow progressive motility) appears to be a common consequence arising from RF-EMR exposure (Fejes *et al*., 2005; Gorpinchenko *et al*., 2014) and was observed in 11/15 studies, as presented in Table 1.

Nevertheless, these studies must be considered alongside others in which the presence of RF-EMR had no overt effect on either progressive (Tas *et al*., 2014) or overall sperm motility (Aitken *et al*., 2005; Imai *et al*., 2009; Trosic *et al*., 2013). A possible explanation for such inconsistencies in the effects of RF-EMR on sperm motility rests with the use of different exposure conditions. Indeed, in a majority of studies reporting negative impacts of RF-EMR on sperm motility (64%), the study design featured the use of isolated human spermatozoa that were exposed to RF-EMR via a mobile phone device. In contrast, at least half of the instances in which no effect was recorded on sperm motility, the studies involved whole-body animal exposure using a signal generator to produce the RF-EMR (Aitken *et al*. 2005; Tas *et al*. 2014; Trosic *et al.,* 2013). While these data further lend support to our proposal of spermatozoa as a sensitive model, they also highlight that *in vivo,* the body may be capable of absorbing some of this radiation (Figure 1); thus diminishing the level of exposure experienced by spermatozoa within the reproductive system.

Effects of RF-EMR on spermatogenesis

In addition to the studies indicating the RF-EMR can have detrimental effects on sperm function, there are sporadic reports that this type of radiation can also affect the testes. It has been demonstrated that a 60 minute exposure of male rats to RF-EMR daily for two weeks can cause widening of the seminiferous tubules (Al-Damegh, 2012). In contrast, Dasdag and colleagues (1999) documented a thinning of seminiferous tubules in response to an intermittent mobile phone exposure of three minutes (on and off) for 2 hours per day in active talk mode every day for one month. To add further difficulty to the interpretation of these data, a subsequent study by the same authors (Dasdag *et al.,* 2003), reported no changes to testis structure following a similar RF-EMR exposure time of 20 minutes every day for one

month. In addition to potential impacts on the diameter of the seminiferous tubules, chronic exposure (3 hours per day for one year) of rats to RF-EMR reportedly elicited a reduction in the thickness of the tunica albuginea (Tas *et al*., 2014). Prolonged exposures (6 hours daily over a 100 day period) have also been associated with patterns of sperm aggregation that were absent from unexposed rats and independent of any impact on sperm morphology (Yan *et al*., 2007). Nevertheless, abnormal sperm morphology arising from RF-EMR exposure has been documented (Wdowiak *et al*., 2007). In humans, these abnormalities have primarily been associated with the sperm head leading to a reduced capacity to engage in interactions with the oocyte (Falzone *et al*, 2010). Curiously however, Ozlem Nisbet *et al*., (2012) suggest that this form of insult appears to have no effect on the head morphology of rat spermatozoa at a frequency of 900 MHz, but instead alleviates the incidence of tail abnormalities and promotes a suite of positive functional outcomes, including increased testosterone levels and superior progressive motility. Furthermore, this group observed better formed seminiferous epithelia with 1800 MHz exposure that was not seen in 900 MHz or unexposed treatments. Moreover, another study involving exposure during pubertal development documented RF-EMR to induce no changes to the spermatogenic cycle or testicular morphology (Tumkaya *et al*., 2013).

Notwithstanding the conflicting nature of the data documented above, recent meta-analyses performed by Adams *et al*. (2014) and Liu *et al.* (2014) have concluded that RF-EMR has two major negative impacts on sperm function: significant reductions in motility and a loss of viability. In line with the recent studies of Mailankot *et al*., (2009) and Trosic *et al*., (2013), this analysis confirmed that sperm concentration is not significantly impacted by RF-EMR treatment. While these

data suggest that RF-EMR is not capable of causing major disruptions to the spermatogenic cycle, in line with Sommers (*et al*., 2009), they do nonetheless highlight an impact on the functional attributes of spermatozoa. Such findings are particularly concerning given that they are attributed, at least in part, to studies involving human spermatozoa and therefore bring into question whether RF-EMR may be having any negative impact on fertility in our species. Collectively, the uncertainty surrounding the effects of RF-EMR on the male germ line presents a challenge for interpretation, which is further exacerbated by the lack of any consolidated, mechanistic explanation for the effects of such low-energy radiation on biological systems.

3. Molecular mechanisms of RF-EMR action

Here, we focus on studies documenting effects of RF-EMR on biology, with the purpose of identifying common pathways that may direct our understanding of how this factor influences biological systems. Furthermore, unveiling a mechanism to explain the biological stresses of RF-EMR will allow us to then rationally assess the clinical relevance of certain exposure conditions.

3.1 Generation of oxidative stress

It has previously been hypothesised that the biological effects of EMR could be attributed solely to heat stress, which is induced at the higher intensities of approximately ≥4 W/kg radiation used in some studies (Hossmann & Hermann 2003; Li *et al*., 2007). However, through the use of various ‘intermittent’ exposure systems (e.g. 5 minutes on / 10 minutes off), it has been demonstrated that the effects of bulk

heat stress are likely to be negligible at the intensities of radiation generated during typical RF-EMR exposure (Liu *et al*., 2013a). Such results have subsequently been verified in the transformed GC2 mouse spermatocyte cell line, where it was shown that such transient exposure patterns are capable of inducing DNA fragmentation and oxidised base adduct formation (Duan *et al*., 2015; Liu *et al*., 2013b) in the absence of a significant impact on temperature.

RF-EMR treatment is known to have the capacity to induce oxidative stress, characterised by excessive generation of reactive oxygen species (ROS) that overwhelm the intrinsic cellular antioxidant capacity, in a variety of tissue types. Indeed, this phenomenon has been documented following RF-EMR treatment in *Drosophila* whole body and ovarian tissue models (Manta *et al.,* 2014), mouse fibroblasts (Hou *et al.,* 2014), cultured breast cancer cells (Kahya *et al.,* 2014), rat heart tissue (Ozguner *et al.,* 2005), human lens epithelial cells (Yao *et al.,* 2008), and mammalian spermatozoa (Agarwal *et al*., 2009; De Iuliis *et al.,* 2009a; Kesari *et al*., 2011). We have also replicated this response using transformed male spermatogonial and spermatocyte germ cell lines; documenting an increase in ROS of mitochondrial origin (B Houston & R J Aitken 2015, unpublished observations). Furthermore, of the 27 RF-EMR exposure studies summarised in Table 1, at least 21 of these (78%) document negative effects of RF-EMR on one or more parameters of sperm function and/or testicular histology that are characteristic of responses elicited by oxidative stress; such as lipid peroxidation, impaired motility and the formation of oxidative DNA damage.

Such pronounced effects on the male germ line may stem from the fact that spermatozoa are uniquely susceptible to oxidative stress. This vulnerability arises due to the highly specialised structure of the spermatozoon, featuring limited

protective antioxidant capacity due to a diminutive cytoplasmic volume and, at the same time, an abundance of substrates for free radical attack including DNA, thiol-rich proteins and polyunsaturated fatty acids (PUFA) (Aitken *et al*., 2012a). The latter are of critical importance to the spermatozoon and are required to generate the membrane fluidity needed to support both motility and the membrane-fusion events associated with fertilization (Lenzi *et al*., 2000). Yet when peroxidised, PUFA elicit the formation of small molecular mass, electrophilic aldehydes that perpetuate a state of oxidative stress (Aitken *et al*., 2012a) as detailed below (Figure 2).

Human spermatozoa exposed to RF-EMR exhibit significant increases in mitochondrial and cytosolic superoxide formation (De Iuliis *et al.,* 2009a; Agarwal *et al*., 2009), as well as a significant reduction in sperm motility (Fejes *et* al., 2005; Gorpinchenko *et al*., 2014). The causative link between excess ROS production and sperm motility loss is a well-established paradigm in sperm biology (Figure 2). This is commonly attributed to increased lipid peroxidation and the ensuing formation of electrophilic aldehydes such as malondialdehyde, 4-hydroxynonenal (4HNE) and acrolein which are capable of covalently binding to proteins, thus compromising their function (Jones et al., 1979; Koppers *et al*., 2008, 2010; Aitken *et al*., 2012a, b; Moazamian *et al*., 2015). In the case of sperm motility, these compounds appear to alkylate sperm axonemal proteins that regulate sperm motility, particularly dynein heavy chain (Baker *et al*., 2015; Moazamian *et al*., 2015). In addition, electrophiles such as 4HNE are also known to promote oxidative stress by stimulating ROS generation through the sperm mitochondria (Figure 2). This situation arises because another group of proteins alkylated by 4HNE are the constituents of the mitochondrial electron transport chain (ETC), particularly succinic acid dehydrogenase (Aitken et al., 2012b). When these proteins become adducted by

4HNE, it promotes the leakage of electrons from the ETC which are then consumed by the universal electron acceptor, oxygen, to generate superoxide anion (Aitken *et al*., 2012b). Via such mechanisms, even slight increases in ROS induced by RF-EMR have the potential to become amplified through the mediation of the mitochondria. In support of this mechanism it has been revealed that RF-EMR-induced ROS production does encourage lipid peroxidation in spermatozoa (Al-Damegh, 2012; Kesari *et al*., 2011). Moreover, lipid peroxidation has also been localised within the testicular and epididymal microenvironments following RF-EMR treatment *in vivo* and this has, in turn, been associated with a loss of sperm motility (Mailankot *et al*., 2009).

If RF-EMR is responsible for the induction of oxidative stress, we should see evidence of ROS overwhelming the sperm cell's antioxidant defences under these conditions (Gharagozloo & Aitken, 2011). Indeed, intracellular concentrations of glutathione peroxidase and superoxide dismutase have been shown to be compromised in the spermatozoa of RF-EMR exposed rats (Kesari *et al*., 2011). Furthermore, the addition of exogenous antioxidants such as vitamin C or E has been shown to significantly diminish RF-EMR induced lipid peroxidation, while simultaneously leading to a partial restoration of the glutathione content of the testis in RF-EMR exposed rats (Al-Damegh, 2012). As an extension of this work, both spermatozoa (Kesari *et al*., 2011) and testes (Al-Damegh, 2012) respond by increasing catalase activity following exposure to EMR. This potentially represents a physiological response aimed at counteracting increases in hydrogen peroxide and other ROS formation induced by RF-EMR stress. Interestingly, it has been suggested that RF-EMR may have more pronounced effects in poor quality spermatozoa as revealed in studies where only a proportion of the sperm population

was found to respond to RF-EMR treatment (De Iuliis *et al*., 2009a). If this were the case then the increased ROS production generated in these highly vulnerable cells, could reasonably be expected to impose an oxidative stress environment upon the remainder of the sperm population (Tosic & Walton, 1950).

Downstream of lipid peroxidation, oxidative stress is known to culminate in oxidative damage to sperm DNA (Figure 2). This has been characterised by elevated levels of the DNA damage marker, 8-hydroxy, 2′-deoxyguanosine (8OHdG; Aitken *et al*., 2012b, c; Aitken *et al*., 2014). Accordingly, RF-EMR exposure has been shown to elicit a significant increase in the staining intensity for this marker in human spermatozoa (De Iuliis *et al*., 2009a). RF-EMR has also been correlated with DNA strand breakage in spermatozoa (Zalata *et al*., 2015), cultured spermatogonia (B Houston & R J Aitken 2015, unpublished observations) and spermatocyte cells (Liu *et al*., 2013a). In the latter cell type, the DNA damage was successfully ameliorated by co-incubation of the cells with the antioxidant, melatonin (Liu *et al*., 2013a). Meanwhile, the observation that RF-EMR has the potential to generate sperm DNA damage is especially concerning due to the fact that these cells are capable of harbouring a considerable oxidative DNA damage load independent of any pronounced effects on motility (Aitken *et al*., 1998). These spermatozoa therefore have potential to participate in fertilisation, whereupon the oocyte would bear the responsibility for repairing the DNA prior to the initiation of S-phase of the first mitotic division. The fact that oocytes are relatively deficient in the first enzyme in the base excision repair pathway, OGG1 (Lord & Aitken, 2015), means that any 8OHdG brought into the egg by the fertilizing spermatozoon are likely to persist into the first cleavage division. Since 8OHdG lesions are potentially mutagenic, these

considerations may carry implications for the mutational load subsequently carried by the offspring, if the father's germ line has been oxidatively damaged by RF-EMR.

The ability of RF-EMR to induce damage which leads to negative biological outcomes is yet to reach consensus, nevertheless, biological effects of RF-EMR are more strongly demonstrated in the literature and are likely to depend on the properties of the affected macromolecule. With respect to proteins, it is expected that this form of damage could be resolved upon turnover, or degradation. However, in the case of long-lived molecules such as DNA, the impact of such damage could be far more insidious. This is particularly the case in the male germline where the integrity of the paternal genome has direct implications for future generations. Of particular concern is the potential for the damage to be acquired in post-meiotic germ cells, which have limited DNA repair mechanisms and are therefore unequipped to resolve the damage. This has been shown previously in spermatozoa, by the existence of dominant lethal mutations (Singer *et al*., 2006), which indicate the possibility of these mutations to be transferred through one generation. Given the strong paradigm for oxidative stress as a key mediator of sperm quality and that published data supports the conclusion that RF-EMR can drive ROS production in the male germ-line, understanding how RF-EMR induces ROS is therefore of key importance.

3.2 Metabolic pathways activated by RF-EMR

It has been demonstrated that RF-EMR has the ability to stimulate signalling pathways in somatic cells, such as those associated with the extracellular signal-regulated kinase (ERK) cascade (Friedman *et al.*, 2007) or heat shock protein

response (Di Carlo *et al.,* 2002; Li *et al.,* 2007; Valbonesi *et al*., 2014). Since both of these pathways are known to be redox regulated it is possible that RF-EMR activates these signal transduction cascades as a secondary consequence of ROS production (Christman *et al*., 1985; Nahomi *et al*., 2015; Polla *et al*., 1996). As indicated above, the major site of intracellular ROS generation observed following RF-EMR exposure are the mitochondria.

There are several lines of evidence that point to the mitochondria being the major mediator of RF-EMR action of biological systems. Thus, in pancreatic cancer cells it has been shown that EMR has the ability to induce extensive changes to the morphology of the mitochondria, stimulating a loss of their membrane potential and significantly increasing production of ROS (Curley *et al*., 2014). This effect is mirrored across a variety of additional somatic cell types including rat hippocampal slices where EMR evokes substantial changes to mitochondrial morphology (Zhao *et al*., 2012) and membrane potentials (Tattersall *et al*., 2001), and human peripheral blood monocytes where it induces a transient decrease in mitochondrial membrane potential that is accompanied by increased ROS production and caspase activation; the latter of which are hallmarks of an apoptotic cascade (Lu *et al*., 2012). As indicated above there is also very clear evidence that RF-EMR activates mitochondrial ROS generation in spermatozoa (De Iuliis *et al*., 2009a).

While such effects of RF-EMR have been recorded at radiofrequency levels of around 900-1800 MHz, corresponding to that emitted by mobile phones (Marchionni *et al*., 2006), contradictory stimulatory effects have in fact been observed at very low frequencies, less than 100 MHz (Marchionni *et al*., 2006; Iorio *et al*., 2011). Indeed, in marked contrast to the negative effects of RF-EMR, extremely low frequency EMR (50 Hz) has in fact been shown to encourage sperm motility (Iorio *et al*., 2011). This

412 effect is also believed to be a consequence of altered mitochondrial activity, however
413 in this instance it appears that the EMR exposure leads to an increase in
414 mitochondrial membrane potential (Iorio *et al*., 2011). Such a discrepancy may be
415 explained, at least in part, by the variable degree of penetration achieved with EMR
416 of different wavelengths (Lin, 1976; Figure 1). In this context, it is well-established
417 that the intensity of the RF-EMR decays exponentially as it penetrates the skin, while
418 penetration depth varies between different tissues and organs (Figure 1; De Iuliis *et*
419 al., 2012; Markov & Grigoriev, 2015). This radiation exposure generally depends on
420 emitted power, but to some extent also depends on other parameters such as the
421 frequency, antenna position relative to the body, and the material properties of the
422 absorbing tissue (Balzano*,* 1999). In any case, the biophysics involved in these types
423 of interactions is unresolved, and represents a major limitation regarding RF-EMR
424 studies (Lerchl, 2013). We have also observed subtle variations in the response to
425 RF-EMR when assessing mitochondrial function in male germ cells at different
426 stages of maturation, with vulnerabilities to RF-EMR appearing to be dependent on
427 the stage of development (B Houston & R J Aitken 2015, unpublished observations).
428 This again highlights the potential difficulties with interpreting and rationalizing the
429 effects of RF-EMR on biology, given the diversity of cells that are potentially exposed
430 by mobile phone use.

431 It is also probable that the variation in mitochondrial membrane potential
432 stimulated by EMR is dependent on SAR, as extremely low intensity radiation (2.5 x
433 10^{-5} W/kg) fails to alter mitochondrial membrane potential in human pro-myelotic
434 leukaemia cells (Jin *et al*., 2012). Similarly, mitochondrial membrane potential also
435 remains unaffected when exposed to low doses of EMR (150-570 μW/cm^2) in mouse
436 endometrial glandular cells, but is successfully impaired with higher intensities (1400

μW/cm^2) (Liu *et al.*, 2012). In human spermatozoa evidence of mitochondrial ROS generation was evident at SAR values above 2.8 W/kg (De Iuliis *et al.,* 2009a), although there are no data linking such ROS generation to a change in mitochondrial membrane potential. Nevertheless, an increase in ROS generation has been consistently reported in studies focusing on the impacts of RF-EMR on spermatozoa (Al-Damegh, 2012; Agarwal *et al*., 2009; De Iuliis *et al*., 2009a; Kesari *et al*., 2011).

It should be noted that within the electron transport chain small concentrations of superoxide are a normal byproduct of this essential redox process. However, the magnitude of ROS leakage varies between the ETC complexes, with Complex I (NADH oxidase) responsible for a bulk of the superoxide, also varies with the substrate utilized for energy production, as observed in isolated mitochondria (Quinlan *et al*., 2013). It is also important to note that superoxide production at Complex I is much more damaging than at Complex III in spermatozoa, due to the mode of emigration of ROS from complex I; to the matrix, allowing for subsequent peroxidative damage (Koppers *et al*., 2008). Meanwhile, ROS generated at Complex III escapes to the intermembrane space, where it encounters the pool of mitochondrial antioxidant protection. The movement of electrons through the electron transport chain is a highly regulated process, partly to limit the production of deleterious amounts of ROS. Perturbation of the electron flow through this chain by RF-EMR, and the subsequent promotion of electron leakage within the mitochondria, would provide a gateway for the formation of ROS such as the superoxide anion (Martino & Castello, 2009) as part of a two-step process (Figure 3). Considering RF-EMR specifically promotes mitochondrial ROS production (De Iuliis *et al*., 2009a; Burlaka *et al*., 2013) associated with increased expression of mitochondrial apoptotic markers (Liu *et al*., 2015) and decreased mitochondrial membrane potential (Lu *et*

462 *al*., 2012), we propose that this radiation potentiates the leakage of electrons within
463 the electron transport chain. Such electron leakage may be achieved through
464 interference with proton transmission through the transmembrane complexes of the
465 inner mitochondrial membrane. This is caused by the ability of modulated EMR (such
466 as that emitted from mobile phones) to augment the oscillation of ions, interfering
467 with their transport through membrane proteins; thus potentially perturbing the strict
468 membrane potentials (Panagopoulos *et al*., 2000; 2002; 2015) enforced in the
469 specific intermembrane compartments of the mitochondria, which otherwise stabilize
470 proton flow (Figure 3; Perry *et al*., 2011). A consequence of reduced proton
471 emigration is a reduced proton motive force and a subsequent reduction in ATP
472 production (Perry *et al*., 2011). Under these conditions, when the $NADH/NAD^+$ ratio
473 is high and associated with low or compromised mitochondrial respiration, as
474 previously shown to be induced by EMR (Sanders & Joines, 1984), superoxide is
475 formed at Complex I (Kudin *et al*., 2004; Murphy, 2009). This scenario is
476 accompanied by the ability of RF-EMR treatment to significantly impair the
477 conformation of proteins and DNA, including key antioxidant proteins (Lu *et al*.,
478 2012), preventing them from participating in the elimination of radicals generated
479 during respiration. Thus, as a first step, the combined effects of RF-EMR results in
480 an imbalance of free radical formation and antioxidant status, driving a state of
481 oxidative stress (Figure 3). The ROS formed through this process, modified to
482 hydrogen peroxide via mitochondrial superoxide dismutase, would in turn have the
483 ability to drive a lipid peroxidation cascade (Al-Damegh, 2012), resulting in the
484 production of electrophilic aldehydes including malondialdehyde (Kesari *et al*., 2011;
485 Mailankot *et al*., 2009) and 4HNE (Moazamian *et al*., 2015). Once formed, these
486 potent electrophiles activate the second step of this response; inducing widespread

interference within the electron transport chain by directly alkylating key proteins associated with the protein complexes of this pathway. As aforementioned, Complex II (succinate dehydrogenase) of this chain is preferentially targeted by 4HNE (Aitken *et al*., 2012b). Modification or inhibition of Complex II prevents oxidation of FAD in the succinate dehydrogenase-A subunit, forcing the flow of electrons to oxygen and thus resulting in elevated mitochondrial perturbation with consequential increases in superoxide formation (Zhang *et al.,* 1998; Aitken *et al*., 2012b). Moreover, since mitochondria are responsible for a majority of ROS production within spermatozoa, (Koppers *et al*., 2008) it is conceivable that disrupting the function of these organelles accounts for the elevated ROS production observed with RF-EMR treatment in several studies, as exemplified by De Iuliis *et al*. (2009b). An important feature of this putative mechanism is that it would account for the subtle or variable changes that RF-EMR has been recorded to induce in terms of sperm motility, owing to the fact that in species such as the human, mouse and rat the energy demands required to support motility are not exclusively dependent on oxidative phosphorylation (Storey, 2008; Williams & Ford, 2001). However, it should be taken into account that these cells are susceptible to a state of oxidative stress.

4. Conclusion

To date, contradictory studies surrounding the impacts of RF-EMR on biological systems maintain controversy over this subject. Nevertheless, research into the biological responses stimulated by RF-EMR is particularly important given our ever-increasing use of mobile phone technology. While clinical studies are identifying possible detrimental effects of RF-EMR, it is imperative that mechanistic studies are conducted that elucidate the manner in which RF-EMR perturbs biological function, thus supplying a rational cause. A focus on the male reproductive

system is justified given the potentially elevated levels of exposure this system may experience as consequences of the personal storage of mobile devices, the unique vulnerability of the highly specialised sperm cell, and the future health burden that may be created if conception proceeds with defective, DNA-damaged spermatozoa. While this subject remains a topic of active debate, this review has considered the growing body of evidence suggesting a possible role for RF-EMR induced damage of the male germ line. In a majority of studies, this damage has been characterized by loss of sperm motility and viability as well as the induction of ROS generation and DNA damage. We have therefore given consideration to the potential mechanisms through which RF-EMR may elicit these effects on spermatozoa, which we utilized as a sensitive model system. We propose a mechanistic model in which RF-EMR exposure leads to defective mitochondrial function associated with elevated levels of ROS production and culminates in a state of oxidative stress that would account the varying phenotypes observed in response to RF-EMR exposure. With further complementary data, this model will provide new impetus to the field and stimulate research that will allow us to confidently assess the reproductive hazards of mobile phone usage.

Conflict of Interest

The authors declare no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure Legends

Table 1. Review of studies investigating the effects of RF-EMR on the spermatozoa and male reproductive system of mice, rats and humans.

Figure 1. Physical aspects of radiofrequency-electromagnetic radiation. A table identifying the estimated intensity of radiation emitted from devices in talk mode of either 900 or 1800 MHz (Durney, 1986; Liu *et al*., 2013; Panagopoulos *et al*., 2010) and plot of penetration depth of this radiation in different tissue types over the MHz-GHz ranges (Gabriel *et al*., 1996).

Figure 2. Oxidative stress cascade within the spermatozoon. ROS is formed within the cell from a variety of possible sources including mitochondrial dysfunction, plasma membrane NADPH oxidase activity, infiltrating leukocytes and environmental factors such as electromagnetic radiation. In the event these ROS outweigh the poor antioxidant capacity of the cell, or a deficiency in this protection exists, a state of oxidative stress ensues. ROS, particularly hydrogen peroxide, attack the lipid membranes which are richly bestowed with polyunsaturated fatty acids that are susceptible to oxidative attack, resulting in the formation of small, reactive aldehydes – acrolein, malondialdehyde and 4-hydroxynonenal. While these aldehydes differ in their reactivity (Moazamian *et al*., 2015) they each target a specific subset of protein centres, typically thiol constituents, as a form of nucleophilic attack. One major consequence of this is impairment of protein function, such as key proteins involved in sperm motility. Succinate dehydrogenase, a protein complex within the mitochondria is a predominantly vulnerable target of these electrophilic aldehydes and alkylation of this complex results in disruption to redox regulated metabolism within the mitochondria, forcing electron flow to oxygen and thus forming yet more

superoxide anion. Furthermore, this imbalance of ROS leads to oxidative DNA damage as hydrogen peroxide migrates to the sperm head and preferentially targets guanine residues within the sperm DNA, highlighted by significant increases in the oxidized base product 8-hydroxy-2'-deoxyguanosine.

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Table 1. Review of studies investigating the effect of RF-EMR on the spermatozoa and male reproductive system of mice, rats and humans.

Reference	Species	Frequency (MHz)	Duration of exposure	Specific absorption rate (W/kg)	Motility	Vitality	ROS	DNA damage	Main outcomes
No effects									
Dasdag *et al.*, 2003	Sprague-Dawley rat	900	20 m per day, 4 weeks	0.52	NA	NA	NA	NA	No effects on testicular structure or sperm morphology
Imai *et al*., 2011	Sprague-Dawley rat	1950	5 h per day, 5 weeks	0.4	NA	NA	NA	NA	No changes to epididymal or testis weights, increased sperm production in EMR treated
Nisbet *et al*., 2012	Wistar rat	900/1800	2 h per day, 90 days	1.2-3/0.01-0.05 (900/1800)	-	NA	NA	NA	Increased sperm motility and morphology with EMR treatment
Sommer *et al*., 2009	C57BL mouse	1966	24 h per day, 4 generations	0.08-2.34	NA	NA	NA	NA	No changes to sperm morphology, count, testis or epididymal weights.
Trosic *et al*., 2013	Wistar rat	915	1 h per day, 2 weeks	0.6	-	NA	NA	NA	No changes to motility, morphology or counts with EMR treatment
Tumkaya *et al*., 2013	Sprague-Dawley rat	900	1 h per day, 45 days	0.48	NA	NA	NA	NA	No effects on testicular size, histology or spermatogenesis
Effects of RF-EMR									
Liu *et al*. 2013	Cultured mouse spermatocyte	1800	1 m per 20 m, 24 h	0.13	NA	NA	NA	+	Increased DNA single strand breaks with radiation intensity which was prevented with antioxidant pre-treatment
Agarwal *et al.*, 2009	Human spermatozoa	850	1 h	1.46	+	+	+	-	Healthy semen donors and infertility patients both experienced a loss in motility, vitality coupled with increases in ROS production. Infertility patients experienced a decreased antioxidant status
De Iuliis *et al.*, 2009	Human spermatozoa	1800	16 h	1	+	+	+	+	Dose dependent effects for all parameters. At 1 W/kg significant decreases in motility and vitality, increases in ROS and DNA damage
Erogul *et al*., 2006	Human spermatozoa	900	5 m	NA	+	NA	NA	NA	Reduced rapid and slow progressive sperm motility
Falzone *et al*., 2010	Human spermatozoa	900	1 h	2	NA	NA	NA	NA	Morphological impacts; reduced acrosome and total sperm head sizes as well as zona binding
Fejes *et al*.,	Human	NA	NA	NA	+	NA	NA	NA	Questionnaire for mobile phone usage, duration of mobile phone usage correlated negatively

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2005	spermatozoa								with progressive motility
Gorpinchenko *et al*., 2014	Human spermatozoa	900/1800	5 h	NA	+	-	NA	+	Reduced progressive sperm motility, increased DNA fragmentation
Wdowiak *et al*., 2007	Human spermatozoa	NA	0-2 years use of phone	NA	+	NA	NA	NA	Reduced sperm motility and increased irregular morphology
Zalata *et al., 2015*	Human spermatozoa	850	60 m	NA	+	NA	NA	+	Significant reductions to sperm motility of men with asthenospermia and oligospermia, significant induction of DNA damage in sperm from healthy and sub-fertile semen profiles
Liu *et al*., 2015	Sprague-Dawley rat	900	2 h per day, 50 days	0.66	NA	NA	+	NA	Decreased epipidymis:body weight ratio, sperm count, and total antioxidant capacity. Increased ROS concentration, apoptosis, ultrastructural neck deformations
Yan *et al*., 2007	Sprague-Dawley rat	1900	6 h per day, 18 weeks	1.8	+	+	NA	NA	Significantly reduced sperm motility and vitality, abnormal sperm clumping
Aitken *et al*., 2005	Swiss mouse	900	12 h per day, 7 days	0.09	-	-	NA	NA	No changes to motility, vitality, concentration or morphology with low SAR and duration. However, degradation to sperm mitochondrial genome
Al-Damegh, 2012	Wistar rat	900/1800	60 m per day, 14 days	0.9	NA	NA	+	NA	Antioxidant treatment prevented seminiferous tubule widening and reduced the lipid peroxidation onset by EMR treatment
Bin-Meferij & El-kott, 2015	Wistar rat	900	1 h per day, 8 weeks	NA	+	+	+	NA	Antioxidant treatment ameliorated a reduction in sperm motility, vitality, count, lipid peroxidation and morphological abnormalities observed with EMR exposure
Dasdag *et al*. 1999	Wistar rat	900	3 m per day, 4 weeks	0.141	NA	NA	NA	NA	Thinning of seminiferous tubules, decreased progression of spermatogenesis. However, potential temperature influences

Ghanbari *et al*., 2013	Wistar rat	915-950	8 h per day, 2-3 weeks	NA	+	+	+	NA	Time dependent decreases to motility, vitality and antioxidant capacity
Kesari *et al*., 2011	Wistar rat	900	2 h per day, 5 weeks	0.9	NA	NA	+	NA	Decreased glutathione peroxidase, superoxide dismutase, histone kinase expression; increased ROS, lipid peroxidation and apoptosis
Kesari & Behari, 2012	Wistar rat	900	2 h per day, 45 days	0.9	NA	NA	NA	NA	Increased caspase activity, morphological abnormalities; decreased testosterone levels, progeny weight and number
Mailankot *et al*., 2009	Wistar rat	900/1800	1 h per day, 4 weeks	NA	+	NA	NA	NA	Reduced sperm motility, but not sperm count; increased MDA and decreased glutathione content of the testis and epididymis
Ozorak *et al.*, 2013	Wistar rat	900/1800	1 h per day, 4 -6 weeks	0.18	NA	NA	NA	NA	Significantly lower lipid peroxidation and total antioxidant status in the testis with 4 weeks EMR treatment. This change was a significant increase with EMR treatment after 6 weeks
Tas *et al*., 2014	Wistar rat	900	3 h per day, 1 year	0.04	-	NA	NA	NA	Increased morphological defects: tunica albuginea thinning, impaired spermatogenesis. No effects on sperm motility or concentration

NA, not mentioned or conducted in study; +, negative effects documented; -, no effects documented. Table arranged by model species used in study. EMR, electromagnetic radiation; ROS, reactive oxygen species; MDA, malondialdehyde; SAR, specific absorption rate

Cell phone mode	Intensity (W/kg; 0, 10, 30 cm distance)
Standby	0.001
Talk (900 MHz)	0.011, 0.002, 0.003
Talk (1800 MHz)	0.008, 0.0009, 0.0002

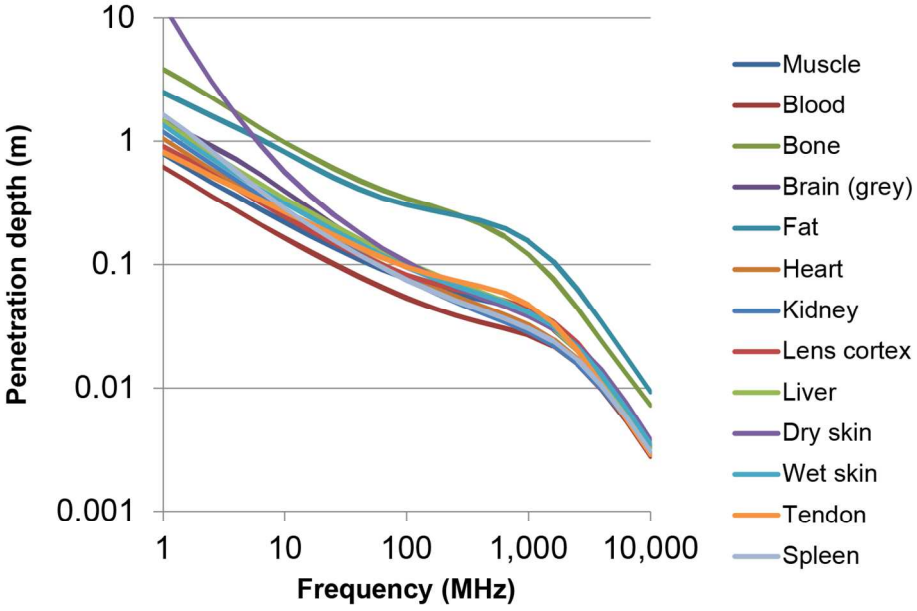

Figure 1. Physical aspects of radiofrequency-electromagnetic radiation. The table identifies typical intensities of radiation emitted from devices in talk mode of either 900 or 1800 MHz (Durney, 1986; Liu et al., 2013; Panagopoulos et al., 2010) and the plot shows the penetration depths of this radiation in different tissue types over the MHz-GHz ranges (Gabriel et al., 1996).

Figure 1
136x130mm (300 x 300 DPI)

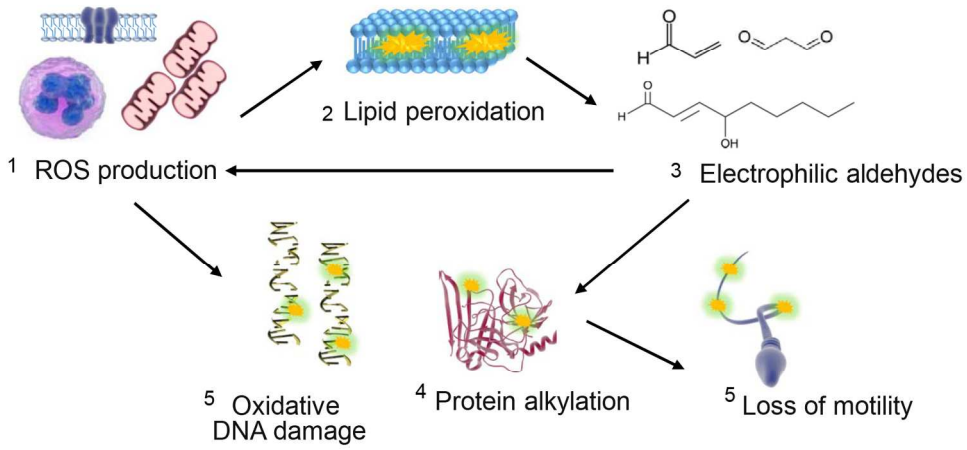

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Figure 2
166x89mm (300 x 300 DPI)

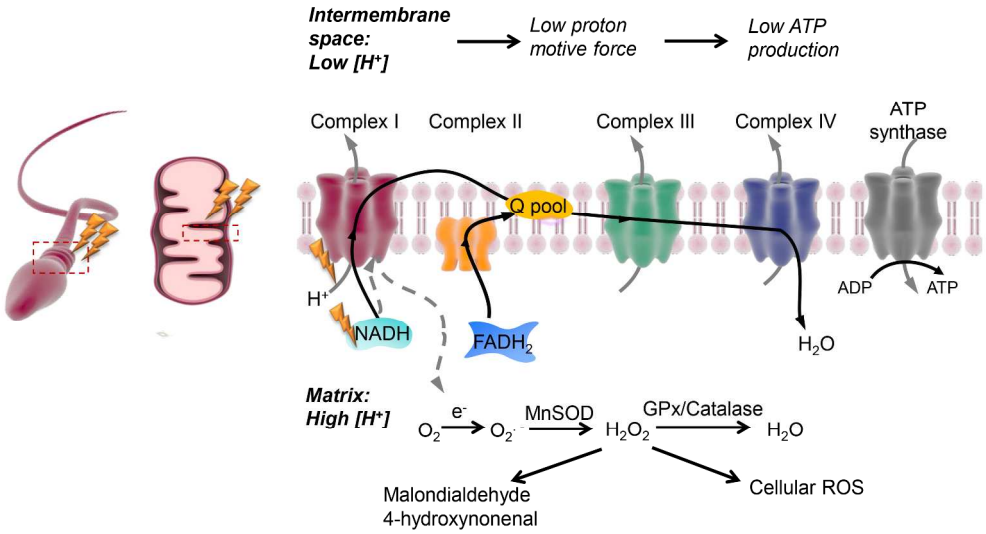

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Figure 3
250x134mm (300 x 300 DPI)